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Rapid Aquatic Toxicity Assay Utilizing Labeled Thymidine Incorporation in Sea Urchin Embryos

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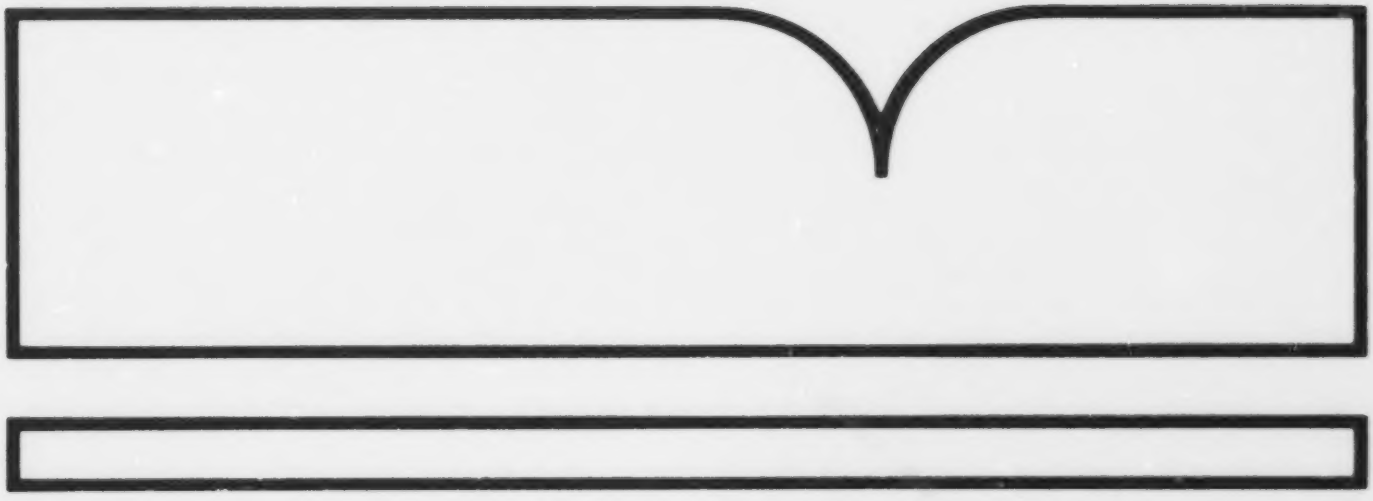

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Eugene Jackim and Diane Nacci

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South Ferry Road
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A RAPID AQUATIC TOXICITY ASSAY UTILIZING LABELED THYMIDINE INCORPORATION IN SEA URCHIN EMBRYOS

EUGENE JACKIM* and DIANE NACCI

Environmental Research Laboratory
U.S. Environmental Protection Agency
South Ferry Road
Narragansett, Rhode Island 02882

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Abstract — Aquatic toxicity was evaluated in the sea urchin embryo (*Arbacia punctulata*) by the inhibition of tritiated thymidine incorporation after a brief exposure to toxic chemicals. In preliminary trials, using eight compounds of varying toxicities, results accurately predicted reduced survival and morphological delay after 48-h exposures ($r^2 = 0.97$). Excellent correlations also exist between these calculated median effective concentrations (EC_{50} values) and literature-derived acute LC_{50} values for two other aquatic species. *Arbacia* is a useful surrogate species for assay: well-studied, easily cultured and fertile virtually year round. The simplicity and speed of this test system lends itself to screening large numbers of compounds, mixtures or water samples.

Keywords — Sea urchin *Arbacia punctulata* Marine embryo Acute toxicity [^{3}H]Thymidine incorporation

INTRODUCTION

With thousands of chemicals and mixtures currently impacting the aquatic environment and new compounds being introduced annually, the task of assessing the toxicity of each substance becomes unwieldy and expensive. Conducting standard acute toxicity tests with aquatic animals requires 24- to 96-h exposures, elaborate facilities and expensive effluent disposal systems.

Embryo tests are fast, inexpensive and reflect sensitivity at a critical life stage [1]. One of the best-documented embryo tests is that with *Crassostrea gigas*, the Pacific oyster, developed for the state of Washington by Woelke [1]. Standard embryo tests have limitations also. Often, embryos of a particular species can be obtained only for a limited time of the year, and difficulties are sometimes encountered in establishing a useful end point. One end point frequently used is the observation of normal development, but this is labor intensive and subjective. Other problems include the impermeability of egg/embryo membranes to toxic chemicals or, in some species, large egg yolks that tend to absorb the test chemical.

The test described here uses *Arbacia punctulata* (sea urchin) embryos. Advantages of this species include year-round availability of gametes, rapid embryo development, small yolks and permeable egg

*To whom correspondence may be addressed.
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membranes. Also, extensive information is available on the embryology, biochemistry and toxicology of this organism [2]. Woelke [1] and Okubo and Okubo [3] produced convincing evidence that the sea urchin and the oyster and many other marine embryos have similar susceptibilities to a wide array of toxic substances. Thus, sea urchin embryos should prove to be good surrogates for assays, with sensitivity comparable to that of many marine and, perhaps as well, freshwater organisms.

By measuring the incorporation of exogenously administered radio-labeled thymidine, DNA synthesis—and thus cell division—is rapidly quantitated in the sea urchin embryo [1,4,5]. Kornofsky and Simmel [6] demonstrated, using autoradiography, that toxic drugs inhibit labeled thymidine incorporation in *Echinarachnius parma* (sand dollar) embryos.

We undertook the present study to develop a rapid surrogate aquatic bioassay. Our assay consists of exposing sea urchin embryos to tritium-labeled thymidine ([^{3}H]-thymidine) in the presence of test substances. Incorporated radioactivity, determined by standard liquid scintillation counting techniques, is used to calculate median effective concentrations. Toxic concentrations are correlated with those determined by standard toxicity tests.

MATERIALS AND METHODS

Arbacia punctulata (sea urchin) were obtained from biological supply companies in Panacea, FL, and Woods Hole, MA. Males and females were held in separate tanks maintained at 15 to 20°C with continuously flowing seawater, and fed *Laminaria* (kelp) *ad libitum*. These culture conditions have produced continuously fertile animals in our laboratory. Eggs from three females and sperm from two males were obtained separately by electrical stimulation using a 10-V transformer [2]. Eggs were washed twice by centrifugation at 100 *g* for 1 min. Sperm concentrations were estimated by optical density at 500 nm. A sperm concentration of 0.8 to 1.0 optical density units was used, representing about 25 million sperm per milliliter.

One milliliter of sperm was added to 50 ml of egg suspension at 20°C and allowed to stand for 10 min. At this time, fertilization was observed under the microscope by the appearance of fertilization membranes. Egg batches with less than 90% fertilization were discarded [7]. Fertilized eggs were again washed twice and centrifuged at 100 *g*.

Seawater at 30.0 ± 1 ‰ salinity was collected in four 16-liter carboys to eliminate daily variability. The water was autoclaved to reduce bacterial contamination and filtered (0.22 μm) prior to use to reduce particulates. The experiments were conducted at 20 ± 1°C.

Washed embryos were counted on Sedwick-Rafter counting chambers and were diluted to approximately 0,000 eggs per milliliter. One hour after fertilization, 1 ml of embryo suspension was added to 99 ml seawater in 70 × 50 mm culture dishes containing the desired concentration of test material. Final embryo density was 100 per milliliter.

Solutions of test compounds (calculated concentrations) were placed directly in the dishes. If the test compound was not water soluble, it was dissolved in dimethyl sulfoxide, ethanol or acetone, so that the final concentration of solvent in test medium did not exceed 0.1%. Solvent controls also contained 0.1% solvent, which were demonstrated to be nontoxic.

The extent of thymidine incorporation was determined by the following procedures. A sample was removed from the control dish to obtain an accurate final egg count. At exactly 2 h after fertilization, 10 ml of the embryo suspension (1,000 embryos) was added to 20-ml glass vials containing 1.52 μCi [^{3}H]thymidine (20 Ci/mmol) in 1 ml seawater, giving a final activity of 0.138 μCi/ml. After 2 h of

thymidine incorporation, the embryo solution was filtered onto glass fiber filters using two 12-filter manifolds. Embryos were washed on the filters sequentially with 5 ml seawater, 5% trichloroacetic acid, 95% ethanol and 1 ml deionized water. Filters were then placed in scintillation vials and treated with 0.1 ml tissue solublizer for 30 min before adding chemiluminescence quenching scintillation cocktail and obtaining counts as disintegrations per minute (dpm).

At 48 h after fertilization, a sample of plutei (normally developed larvae) were counted under a microscope and expressed as percent of the number of original eggs. Any experiment in which control plutei constituted less than 50% of the original egg count was considered invalid.

Data on the incorporation of [^{3}H]thymidine and the number of plutei after 48 h of growth were plotted against the log of the nominal concentrations of test compound. A linear regression analysis was performed on these data [8]. The effective concentrations that induced 50% inhibition of control values (EC_{50} values) were calculated with 95% confidence intervals [9].

RESULTS

The increasing incorporation of [^{3}H]thymidine over 2 h (Fig. 1) reflects growth of the embryo. Typically, growth proceeds from the 4- to the 32-cell stage during the period monitored. Incorporation is inversely related to toxicant concentration.

Figure 2 shows a typical response to increasing concentrations of one of the test compounds, 2-(2-ethoxy-ethoxy)-ethanol. Biochemical data ([^{3}H]thymidine incorporation at 2 to 4 h after fertilization) and morphological data (surviving plutei at 48 h) are plotted against log concentrations of chemical to calculate toxicity values and 95% confidence intervals.

For the experiment shown (Fig. 2), the biochemically derived value, (B)EC_{50}, was 11,720 mg/L (8,656–15,867 mg/L) and the morphologically derived value, (M)EC_{50}, was 7,611 mg/L (5,832–9,917 mg/L).

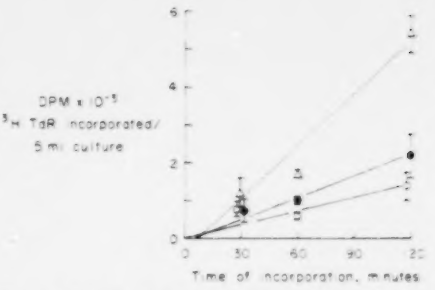

Fig. 1. Effect of 2-methyl-2,4-pentanediol on tritiated thymidine (^{3}H TdR) incorporation over 2 h (mean ± SD): control (triangles); 9,440 mg/L (filled circles); 18,880 mg/L (open circles).

A list of (B)EC_{50} values for compounds of varying toxicities from duplicate experiments is shown in Table 1.

The effects of these eight compounds on *Arbacia* embryos as determined by the two methods described are compared. Figure 3 is a plot of the mean log values for (B)EC_{50} versus (M)EC_{50}. Agreement between respective toxicity values is excellent ($r^2 = 0.9682$). The least-squares regression line is described by the equation: log (M)EC_{50} = 1.0422 [log (B)EC_{50}] − 0.3063. The maximum difference between values is 0.6089 log of the value for 2,4-pentanedione.

Median effective concentrations [(B)EC_{50}] for *Arbacia* embryo were compared with median lethal concentrations (LC_{50}) for two commonly tested aquatic species. Figure 4 compares *Arbacia* with *Daphnia magna*, tested in a standard 48-h static renewal assay (R. V. Thurston, manuscript in preparation, Montana State University Fisheries Bioassay Laboratory). The line describing this relationship is: log *Daphnia* LC_{50} = 1.1290 [log (B)EC_{50}] − 0.9741, with $r^2 = 0.9690$. The results for 2-chloroethanol were most disparate, with differences between values equal to 1.0116 log. This compound is also seen as an outlier in Figure 5, which compares *Arbacia*

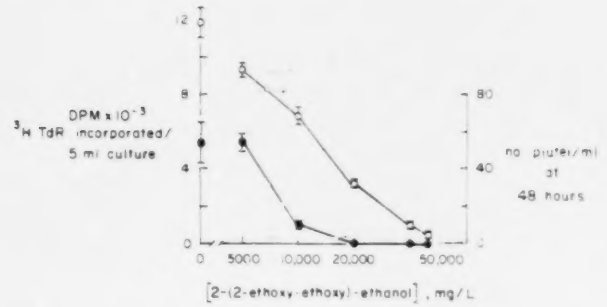

Fig. 2. Effect of increasing concentrations of 2-(2-ethoxy-ethoxy)-ethanol on sea urchin embryos monitored biochemically (open circles; by tritiated thymidine (^{3}H TdR) incorporation from 2 to 4 h postfertilization) or morphologically (filled circles; number of pluteii after 48 h).

with *Pimephales promelas* (fathead minnow), tested in a standard 96-h flow-through assay, (R. V. Thurston, manuscript in preparation).

The correlation coefficient for the model comparing fathead minnow and sea urchin for all eight compounds was 0.8402. If 2-chloroethanol is deleted, $r^2 = 0.9865$ and the line is described by: log *Pimephales* $LC_{50} = 1.1360 [\log (B)EC_{50}] - 0.6708$.

In the *Daphnia* assay and the *Arbacia* incorporation test, all eight chemicals were ranked identically. From least to most toxic, they are 2-methyl-2,4-pentanediol, 2-(2-ethoxy-ethoxy)-ethanol, 2-methyl-1-propanol, 2-chloroethanol, 2,2,2-trichloro-ethanol, 2,4-pentanedione, hexachloroethane and pentachlorophenol. In the fathead minnow comparison, the ranking is similar, but 2-(2-ethoxy-ethoxy)-ethanol is less toxic than 2-methyl-2,4-pentanediol, and 2-chloroethanol is the third most lethal compound.

DISCUSSION

These preliminary trials indicate that the marine embryo incorporation test may be an efficient and sensitive predictor of acute aquatic toxicity for many chemicals.

The assay is based on the principle that the inhibition of thymidine incorporation is proportional to DNA synthesis inhibition

Table 1. Median effective concentrations of eight chemicals as determined by the inhibition of [^{3}H]thymidine incorporation in duplicate experiments using *Arbacia* embryo

Compound	(B)EC_{50}^{a} (mg/L)	95% confidence interval
2-(2-Ethoxy-ethoxy)-ethanol	10.868	7.704 - 15.332
	11.720	8.656 - 15.867
2-Methyl-2,4-pentanediol	13.555	9.757 - 18.832
	9.486	7.266 - 12.385
2-Methyl-1-propanol	2.601	1.859 - 3.646
	2.840	1.558 - 5.183
2,2,2-Trichloro-ethanol	625	474 - 821
	496	296 - 833
2,4-Pentanedione	206	68 - 561
	169	81 - 240
2-Chloroethanol	1.834	1.654 - 1.834
	1.412	862 - 2.309
Hexachloroethane	9.32	8.29 - 10.60
	8.51	7.43 - 9.19
Pentachlorophenol	0.87	0.23 - 3.52
	0.71	0.34 - 1.64

[a]Biochemically derived value.

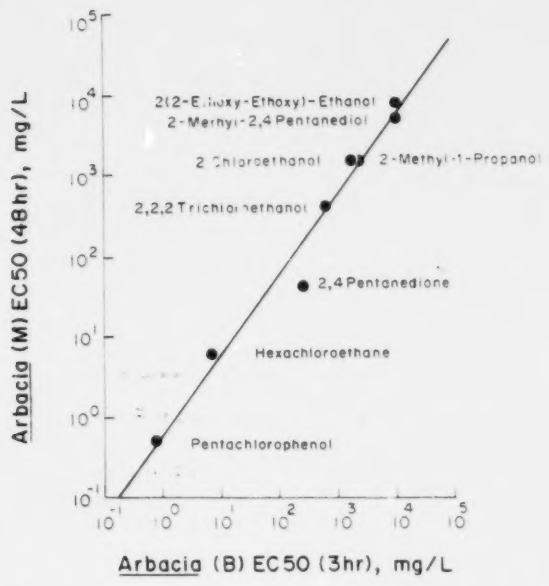

Fig. 3. Comparison of log *Arbacia* (B)EC_{50} values with log *Arbacia* (M)EC_{50} values, described by the least-squares line: $y = 1.0422x - 0.3063$, with $r^2 = 0.9682$.

and thus retardation of cell division. Slowing of normal cell division can be caused by blocking major physiological functions, such as respiration, membrane transport, etc., as well as by directly blocking DNA synthesis. Thus, inhibition can be produced by a spectrum of toxic mechanisms. These test results reflect general toxicological effects predictive of lethality. This was evidenced by the correlation between toxicity values as assessed by two methods in the sea urchin embryo. There is also close agreement in the toxicity rankings of these eight compounds using three different aquatic species, each employing unique test procedures. The exceptional compound is 2-chloroethanol. It is speculated that this compound is metabolized by some species to the more toxic 2-chloroacetic acid [10]. High toxicity in the fathead minnow may reflect this metabolism, while in *Arbacia*, toxicity results agree with those of the parent compound as predicted by structure-activity modelling [10].

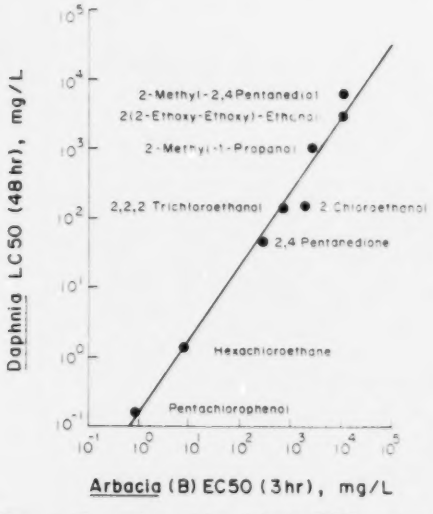

Fig. 4. Comparison of log *Arbacia* (B)EC_{50} values with log *Daphnia* LC_{50} values, described by the least-squares line: $y = 1.1290x - 0.9741$, with $r^2 = 0.9690$.

To its technical advantage, the marine embryo incorporation test is rapid and labor efficient; currently, one person can conduct a 24-sample assay in 1 d. This test also uses small samples (minimizing toxic wastes) and provides an objective, statistically useful end point. Culturing and testing can be done

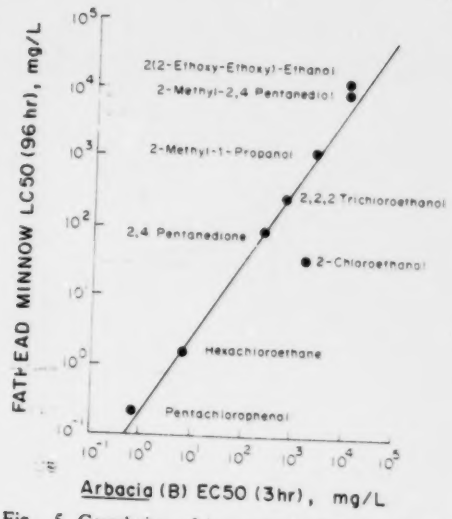

Fig. 5. Correlation of log *Arbacia* (B)EC_{50} values with log fathead minnow LC_{50} values, described by $r^2 = 0.8402$ when all eight compounds are considered. When 2-chloroethanol is treated as an outlier, the line is described by the least-squares line: $y = 1.1360x - 0.6708$, with $r^2 = 0.9865$.

using commonly available laboratory equipment. We are currently limited by the number of samples that can be processed in this timed procedure. The assay is now being semiautomated to increase efficiency, accuracy and productivity.

With this expanded capacity, the relative toxicity of 100 samples could be assessed simultaneously. Potential uses include continued testing of pure compounds to substantiate comparisons between species, evaluation of homologous compounds (effects of functional groups and positions), investigation of new and untested chemicals and toxicity assessment of environmental extracts.

The standard sea urchin larval test (morphological end point) has long been used to assay environmental water quality, wastewaters, etc. [3,11]. The application of this incorporation test could greatly simplify the handling of samples of this type.

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